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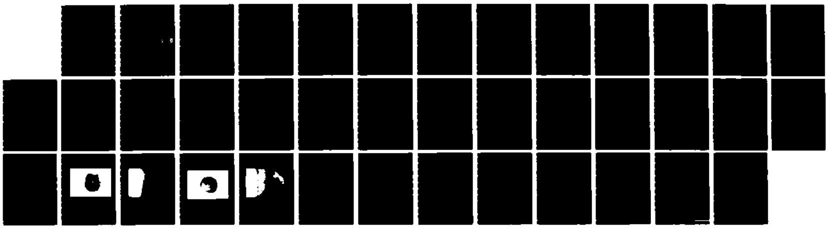

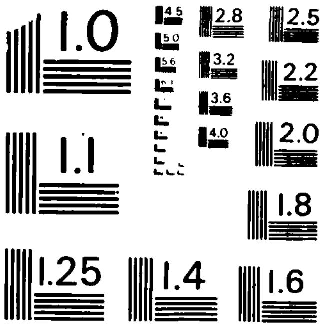
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DERMAL INFLUENCE ON EPIDERMAL RESURFACING DURING THE REPAIR OF SPLIT THICKNESS WOUNDS

ANNUAL REPORT

Patricia A. Hebda, Ph.D.
William H. Eaglstein, M.D.

August 15, 1984

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The purpose of this study is to eveluated dermal-epidermal interrelationships during wound repair by assessing the effect on epidermal wound healing of factors derived from platelets. Two methods were used to approach the problem: 1) a skin explant culture model in which small sections of partial thickness pig skin (explants) were grown in medium containing fetal bovine serum and/or a platelet homogenate, and epidermal cell outgrowth from the explant was measured; 2) a wound

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healing animal model in which partial thickness excisional wounds made on the backs of young domestic pigs were treated with platelet preparations then evaluated for epidermal resufacing and dermal collagen biosynthesis. Our results are as follows:

1. In explant cultures:

   a. Platelet Homogenate Fraction initiated epidermal cell outgrowth without serum.

   b. Platelet Homogenate Fraction stimulated the rate of outgrowth.

   c. The stimulatory factors were stable to heating at 100°C for 2 min.

   d. Commercial PDGF without serum did not support explants. With serum it had a slight stimulatory effect.

   e. Platelet Homogenate Fraction together with serum was able to produce an inhibition of explant viability and outgrowth.

2. In wound healing:

   a. Topical application of Platelet Homogenate (5 min/day for 7 days) did not affect epidermal or dermal healing.

   b. Topical application of Platelet Homogenate with Occlusion (for 2 hr or 6 hr) enhanced epidermal wound healing compared to Air Exposed wounds, but was not better than Vehicle with Occlusion.

Our conclusions are that platelet components (other than PDGF) stimulate epidermal cell viability and outgrowth in explant cultures. We recommend further exploration of these factors. The factors should be purified from Platelets, compared with known growth factors, (Epidermal Growth Factor and Serum Spreading Factor (Epibolin) and evaluated first _in vitro_ with epidermal explant and cell culture systems. They should next be tested in animal wound healing. Finally, an effective treatment should be evaluated in a clinical study with human subjects.

ABSTRACT

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Hebda, P.A. and Eaglstein, W.H. (1984) The Effect of Platelet actors on Epidermal Cell Outgrowth in Skin Explants. (Abstract) J. Invest Dermatol. 82:410 35

PROBLEM

This study focuses on epidermal and dermal interrelationships during wound repair. Blood platelets contain a growth factor for dermal fibroblasts. We have previously shown that platelets also contain a factor or factors that affect epidermal cells in explant cultures. We are characterizing the effects of platelets on the outgrowth of epidermal cells in a skin explant culture model and on the rate of epidermal wound healing in an animal model. The goals of this study are directed towards obtaining knowledge of the wound healing process and the role of platelets to develop more effective ways to treat skin injury.

BACKGROUND AND SIGNIFICANCE

The Skin

The skin is a three-layered covering resting on subcutaneous fat. The outer, highly cellular, thin epidermis is in contact with the dermis by way of multiple, interpapillary ridges (1). These ridges result in increased surface contact between the two layers; they provide much of the resistance of normal skin to tangential stress. The epidermis and the dermis are separated by a continuous acellular basement membrane. The innermost portion of the epidermis is the basal layer containing the cells destined to become the keratinous outer stratum corneum. Cells generated from the basal layer are gradually extruded towards the surface as they undergo differentiation, forming first the stratum spinosum (1), characterized by prominent interlocking cell wall projections which further aid the skin's ability to withstand shearing forces. The next layer is the stratum granulosum which merges imperceptibly with the stratum lucidum. These layers are involved with heat regulation and keratinocytes in these two layers synthesize the waterproof fibrous protein keratin. The keratinocytes mature and die, leaving a keratin sheath which makes up the outermost layer, the stratum corneum. The epidermis, especially the stratum corneum, is the body's primary defense against penetration by noxious environmental elements and also prevents fluid loss.

The underlying dermis, which is primarily collagen, elastin and proteoglycans, is 20 to 30 times thicker than the epidermis in human skin and contains the nervous, vascular, lymphatic and supporting structures of the skin, as well as harboring the epidermal appendages. Fibroblasts produce the fibrous proteins, collagen and elastin, which give skin its strength (2). Mast cells containing histamine and heparin produce the proteoglycans (ground substance) (3) which form the interfibrillary matrix of the dermis. Tissue macrophages are distributed around blood vessels and hair follicles (4). The appendages of human skin are the hair follicles and their associated sebaceous glands, the eccrine sweat glands which enter through interpapillary ridges, and the aprocrine glands located primarily in the axillary and inguinal regions (5).

Wound Healing

The wound healing process has been extensively studied. Ideally, the healing process will restore normal structures and may be considered regeneration; in most cases, it does not achieve the original state but repairs the defect. Repair occurs in dermal healing; collagen, glycosaminoglycans and other extracellular components are produced to fill in the defect and restore the contour while angiogenesis restores the blood supply system. Some scarring almost always occurs with repair. Sometimes the repair process is aberrant and excess collagen results in keloids or hypertrophic scarring. Epidermal healing is closer to regeneration because the same original structures are restored, by a sequence of migration, mitosis and maturation of epidermal cells.

In full thickness wounds, contraction accounts for a portion of the healing process. In partial thickness wounds, healing involves both the dermis and the epidermis, but contraction does not occur. While dermal fibroblasts are producing collagen, epidermal cells from the wound margins and in remaining epidermal appendages form a new surface (re-epithelialize) to cover the wound bed by undergoing migration, mitosis and maturation. Dermal and epidermal healing occur simultaneously but an inter-relationship remains to be demonstrated.

Wound Healing and Platelet-Derived Growth Factor

The first responses to skin injury are bleeding, coagulation and inflammation. Blood enters the wound, a clot is formed by activated platelets and fibrin, and specific blood components including platelet-derived growth factors are released at the site of injury. Blood-derived growth factors were first proposed by Balk (6) in 1971. He demonstrated that nontransformed chick embryo fibroblasts grow well in medium with serum, but only virally transformed fibroblasts grow in plasma-supplemented medium. In 1974, Ross and co-workers (7) and Kohler and Lipton (8) showed that platelets are the source of most of the mitogenic activity of serum. These studies led to the hypothesis that platelets activated at the site of vascular injury release components that trigger the proliferative response (7,9). Studies of this hypothesis resulted in the isolation and characterization of Platelet-Derived Growth Factor (PDGF) which is a potent mitogen for mesenchymal cells, including smooth muscle cells and dermal fibroblasts both key participants in wound healing. PDGF is a heat stable, cationic, reducible protein having a M_r (relative molecular mass) of approximately 30 K (10,11). PDGF has been purified and demonstrates consistent M_r heterogeneity in the carbohydrate side chains which does not affect activity, but which results in two separable native species PDGF I and PDGF II (12,13). PDGF is localized in the alpha granules of platelets and is released during platelet activation. It has many biological effects on connective tissue cells including stimulation of chemotactic activity (14), mitosis (15) and collagen biosynthesis (16,17). Experimental evidence suggests that PDGF is specific for mesenchymal cells; it did not affect epidermal cell outgrowth in our _in vitro_ explant model (see Section C) nor does it stimulate an epithelial cell line (10). Heldin et al. (18) found evidence to indicate that

receptors for PDGF are restricted to connective tissue and glial cells. However, there is a conflicting report by Aso et al. (19) that partially purified PDGF added to plasma-supplemented medium results in an increased size and number of epidermal cell colonies compared with control cultures grown in medium with platelet-poor plasma. Recently it was reported that PDGF is very similar or identical to the product of the simian sarcoma virus V-sis oncogene (20,21). This is the first association between the product of an oncogene and a protein which has a known physiological function in normal cells.

Other Blood-Derived Factors

Several growth factors other than PDGF have been found in platelets (20). Platelet Basic Protein (PBP) and Connective Tissue Activating Peptide III (CTAP III) are the best defined of this group. These two proteins, like PDGF, are cationic, but they are smaller than PDGF, PBP is 14-17 K (22) and CTAP III is about 9.3 K (23). Other platelet factors reported in the literature are a 40 K acidic protein that stimulates glial cells (24), a 30-50 K protein that targets a number of cultured cell lines (including a tumor cell line of epithelial origin) (25) and a 72 K protein, with an isoelectric point between 7.8 and 8.3, that stimulates SV 40 transformed cells (26). None of these has been tested for an effect on epidermal cells.

Other possible candidates for blood-derived wound hormones are Epidermal Growth Factor (EGF) (27), Epibolin (28) and Serum Spreading Factor (SSF) (29). EGF, first isolated from mouse submaxillary gland and later identified in human urine, has been shown to stimulate epidermal cell outgrowth in explant culture of neonatal but not adult skin (30). EGF is a small monomeric protein (6 K), is extremely stable (31) and has been extensively characterized (32,33). It has recently been reported to be associated with serum and platelets (34). Epibolin is a serum and plasma factor that supports epithelial cell spreading and movement in culture. It was purified from human plasma and characterized by Stenn (28). It is a single-chain glycoprotein (65 K) that is heat and trypsin labile. Serum Spreading Factor (SSF) is the name given to two purified components (65 K and 75 K) originally derived from serum but also found tightly associated with platelet membranes (29). Epibolin and 65 K SSF have greater than 70% homology and rabbit anti-human Epibolin cross reacts with human SSF (29). It is very probable that since SSF is tightly bound to platelet membranes it is present in washed platelet preparations and may partition into the PHF currently under investigation.

The studies reported here show that a platelet homogenate fraction (PHF) added to serum-free medium supports epidermal cell outgrowth in explant cultures. This is the first evidence to the investigators' knowledge for support and stimulation of epidermal cell growth by platelets in the absence of other serum or plasma components, and may indicate a "new" platelet-derived factor.

Choice of Experimental Systems

Epidermal cell outgrowth from explants (35) is a good *in vitro* model for re-epithelialization because the tissue structure of the epidermis and dermis is partially retained. Although systemic responses are absent in this system, localized interactions, including fibroblast-epidermal, substratum-epidermal, and basement membrane-epidermal interactions remain.

The porcine wound healing model to be used was developed by the principal investigator (36). This method is an improvement on the porcine epidermal wound healing model of Winter (37). In Winter's model, partial thickness wounds are made on the backs of pigs then daily biopsy specimens are examined for re-epithelialization by serial cross section. The investigators' model permits macroscopic evaluation of epidermal healing. It agrees with Winter's model but is facile enough to permit many precise comparisons between topical treatments. It was subsequently expanded to include an assay of dermal collagen content and biosynthesis according to Diegelmann et al. (38) as a measure of dermal healing. Many different potential methods of improving wound management have been evaluated with this model (36,39-45).

Porcine skin is very similar to human skin, with respect to relative thicknesses epidermis and dermis, relative sparsity of hair and the presence of a layer of subcutaneous fat. The large size of the animal (11-15 kg) permits each animal to serve as its own control since many small wounds can be made on each individual and divided into several treatment groups. Other animal models for wound healing have been reported in the literature. Most of these use rodents such as guinea pigs (46), rats (47), hampsters (48,49), and rabbits (50). Evaluation of wound healing is made by a variety of measurements including collagen deposition in sponge implants (49), histology of serial cross-sections (51), visual assessment (47,50,52) and measurement of tensile strength (46). All of these models have inherent problems and limitations. The structural features of rodent skin are quite different from human skin and the small size of the animal limits the number of wounds which can be made on each animal. In addition, only one aspect of healing is evaluated--either epidermal resurfacing or dermal collagen production.

The experimental design involves a heterologous system in that the factors are derived from human blood and the target tissue is porcine skin. The investigators chose the design based on most readily available materials and because there is no evidence that excludes cross-reactivity between the two species; blood-derived growth factors thus far purified show little species specificity (10). However, *in vitro* experiments with human skin will be included at appropriate stages and compared with results obtained with porcine skin.

Platelet Factors and Wound Healing

Platelet factors (intracellular or cell membrane-bound) are excellent candidates for wound hormones, because platelets are released at the wound site when bleeding occurs. The research discussed in this

report will provide important information about the physiological role of platelets in the healing process. It may indicate a mechanism for the coordinated regulation of dermal and epidermal healing. It may also lead to the development of therapies utilizing exogenous platelet fractions to augment endogenous factors. These results will 1) provide additional evidence for the pivotal role in wound healing ascribed to platelets by defining their effect on epidermal cells, 2) identify the "wound hormones" derived from platelets and 3) offer a possible approach to improve the treatment of burn wounds and some other slow healing or chronic wounds (stasis and decubitus ulcers). A platelet transfusion was successful in inducing healing of a chronic ulcer in a patient with a platelet deficiency (46).

It is also of great interest to learn how platelet-derived factors function in the overall processes of development, growth, regeneration, repair and aging. Once completed, this study, which focuses on repair and regeneration as they occur in healing skin, will permit investigation of other events. Epidermal regeneration and dermal repair may be compared and contrasted with developmental growth to understand why wound healing is an imperfect restorative process. The knowledge gained about the basic biochemistry of the skin may ultimately lead to the development of methods for inducing complete tissue regeneration such as the regrowth of a digit or limb.

APPROACH

The following two methods were used to test for a stimulation of epidermal wound healing by platelets--an _in vitro_ skin explant culture study (35) and an _in vivo_ wound healing study using an animal model (36).

Materials

RPMI 1640 Medium[1], FBS and AB/AM (penicillin 10,000 units/ml, fungizone 25 ug/ml, streptomycin 10,000 ug/ml after rehydration in 20 ml H_2O) were obtained from GIBCO (Grand Island, NY). Chemicals and biochemicals were reagent grade. Commercial PDGF was purchased from Bethesda Research Labs (Bethesda, MD). Domestic Yorkshire swine were supplied by Dalessio's Stock Farm (Plumville, PA). Animals had been raised in confined housing to protect the skin from abrasion and were maintained in the Central Animal Facility (approved by AAALAC) of the University two weeks prior to use. Here they received water and a complete swine grower diet _ad libitum_ and were housed in pens controlled for temperature (20-23°C) and light (12h/12h, light:dark). Human platelets were obtained from the Central Blood Bank (Pittsburgh, PA). The platelets were less than one week old and were physiologically normal.

Platelet Homogenate Fraction. Concentrated human platelets from the Blood Bank (approximately 10 x from whole blood) were centrifuged at 400 x g for 10 min to sediment residual red blood cells. The supernatant platelet-rich plasma was centrifuged at 1500 x g for 10 min to yield a platelet pellet and supernatant platelet-poor plasma. The plasma was poured off and discarded and the packed platelets were gently

resuspended in an isotonic buffer (100 mM KCl, 50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4) (54). This platelet suspension was centrifuged at 1500 x g for 10 min to yield washed platelets. The platelets were resuspended in a small volume of platelet incubation medium (1 ml/unit of platelets) and homogenized with a Tekmar Tissumizer at 10,000 rpm for 30 sec. The homogenate was centrifuged at 15,000 x g for 10 min to sediment cellular debris which was discarded. The supernatant solution was sterilized by passage through a 0.45 u Millex filter (Millipore Corporation). This Platelet Homogenate Fraction (PHF) was assayed for protein concentration with the Bio-Rad rapid protein assay (55) (Bio-Rad), which is based on the method of Lowry et al. (56).

Heat Inactivation of Platelet Homogenate Fraction. PHF was added to serum-free medium at a concentration of 260 ug protein/ml. The suspension was incubated in a boiling water bath ($100^{\circ}C$) for 2 min then rapidly cooled in an ice bath. After filtration through a 0.45 u Millex filter, the heat-inactivated PHF concentrate was added to medium with or without FBS to produce a ten-fold dilution of PHF (approximately 26 ug protein/ml, some denatured protein being lost during filtration).

Methods

A. In Vitro Studies

Skin Explant Culture Model. For each experiment, a skin specimen was excised from the paravertebral region of a young Yorkshire swine (11-15 kg). The animal, which had received no previous anesthesia or treatment, was anesthetized with ketamine (300 mg, i.m.) and metoxyfluorane (3%, open mask). The skin on the back was prepared for excision as follows: Hair was as removed with fine barber's clippers and a razor. The skin was washed sequentially with neutral soap and water, 10% povidone-iodine (1x), 70% methanol (2x) and sterile distilled water (3x). By means of aseptic techniques, 50 x 22 mm skin sheets were excised with a Castro-Viejo electro-keratome set to cut at a depth of 0.2 mm. The sheets were placed in RPMI 1640 medium containing 2% AB/AM and kept at $4^{\circ}C$ prior to dissection. The explants were prepared within 24 hr after surgical excision, according to the methods originally developed by Halprin et al. (57) and adapted to culture of young adult porcine skin by Hammar and Halprin (35). The skin sheets were kept moist with medium throughout the dissection procedure. Each sheet was examined macroscopically and selected for uniform thickness. Individual explants (1-2 mm squares) were prepared with a sharp scalpel taking care to cut clean edges perpendicular to the surface (35). Explants were placed dermal-side down in 35 mm plastic culture dishes, four explants/dish. A minimum of ten dishes/group were prepared for each experiment. After the explants air-dried for 15-30 min to obtain adherence to the plastic culture dish, 2 ml of culture medium containing the appropriate test material and 2% AB/AM was added to each dish. The explant cultures were incubated at $37^{\circ}C$ with 5% CO_2.

Evaluation of explant outgrowth. Cultures were examined for onset of epidermal cell outgrowth. Once outgrowth was observed (Day 3 in these experiments), the longest radius on each growing side of each explant was measured using a phase contrast microscope (100x) fitted

with a micrometer eyepiece. The longest radius for each side was measured again on Days 5 and 7. The change in the radius for each side was calculated and the maximum difference in the radius between Days 3 and 7 was used as the value for the rate of outgrowth for each explant. Mean values and standard errors of the means (SEMs) were then calculated for each treatment group. Student's t-test (58) was applied to test for significant differences in rates of outgrowth between treatment groups. A difference was considered significant when the p value was less than or equal to 0.05. Explant viability was measured as the number of explants showing outgrowth on Day 3 divided by the total number of explants prepared. This fraction was multiplied by 100 and expressed as % viable explants.

B. In Vivo Studies

Animal Wound Healing Model. Experimental details of this procedure may be found in the previous Annual Report (August 15, 1983).

In the studies reported here wounds were treated with platelet homogenates by one of two methods of application: 1) Topical application of a platelet homogenate (3×10^{10} platelets/ml) or vehicle (platelet buffer) to each wound and incubation for 5 minutes before remvoal of excess fluid and 2) Topical application of a platelet homogenate (10 mg protein/ml, approximately 3×10^{10} platelets/ml) or vehicle to each wound and occlusion under a plastic film dressing to keep the treatment on the wounds for several hours (2 or 6) after which time the dressings were removed. In all experiments control wounds which received no treatment were also included.

RESULTS

A. In Vitro Studies

Effect of Platelet Homogenate Fraction on Explant Outgrowth. The explants were cultured in medium containing varying concentrations of PHF and FBS. A total of thirty culture dishes were prepared for each of the eight treatment groups. There were four explants per culture dish yielding a total of 120 explants per group. Equal numbers of explants in all groups were prepared for each individual experiment to avoid introducing differences due to variation in skin and treatment conditions. Onset of outgrowth was observed on Day 3. The measurements of epidermal cell outgrowth on Days 3, 5 and 7 are shown in Table I. Except for Group 1 (negative control) each group produced 26-69% viable explants, that is, showed outgrowth. The % viable explants in Groups 2-4 (PHF alone) ranged between 45% and 69%, which was comparable to Group 5, the positive control of 5% FBS (67%). However, the % viable explants were lower for Groups 6-8 grown with PHF + FBS, 26-37%. Only 2% (two explants) showed outgrowth in medium without PHF or FBS (negative control group), and this outgrowth stopped after Day 5.

The explant outgrowth measurements are plotted in Figure 1 and demonstrate that PHF evoked total outgrowth very similar to, but slightly less than, the outgrowth obtained with 5% FBS ($p = 0.05$). The

outgrowth from explants grown with PHF + FBS was much less than with either supplement alone in both the radius of the epidermal sheet (total outgrowth) and the rate of outgrowth ($p = 0.001$).

In Figure 2, the rates of outgrowth between Days 3 and 7 are shown for different concentrations of PHF either with or without FBS. There was a concentration-dependent increase in the rate of outgrowth with PHF between 26 and 130 ug protein/ml which reached a plateau between 130 and 260 ug/ml. The rates of outgrowth were lower with PHF + FBS than with FBS or PHF alone. This inhibition was not PHF concentration-dependent within the range tested (26-260 ug/ml).

Cultures were examined under a dark field and phase contrast microscope for structural features characteristic of epidermal sheets from explants (35). Cultures grown with PHF (Figure 3A,B) had epidermal sheets similar to FBS controls--the sheet was multilayered near the explant, tapering to a single cell layer at the growing edge with many "stretched" epidermal cells (57). Cultures grown with PHF + FBS (Figure 3C,D) had abnormal features compared with FBS controls. The epidermal sheet was thick and multilayered throughout, there was no tapering toward a monolayer as is normally found. The cells at the leading edge were not "stretched" or spread but were rounded and piled up.

Effect of Platelet-Derived Growth Factor on Explant Outgrowth. Explants were cultured in serum-free medium containing commercial partially-purified PDGF, at concentrations of 0.2, 1.0 and 5.0 units/ml. The results are summarized in Table II. PDGF did not support epidermal cell outgrowth in the absence of FBS. In the presence of 5% FBS, PDGF produced a slight stimulation of epidermal cell outgrowth at 1.0 and 5.0 units/ml (p 0.05); there was not significant stimulation with PDGF at 0.2 units/ml.

Effect of Heat on Platelet Homogenate Fraction. Explants were cultured in media containing heat-treated PHF and outgrowth was evaluated and compared with PHF- and FBS-treated groups. The results, shown in Table III, indicate that PHF was able to support epidermal cell outgrowth after heat treatment and the rate of outgrowth was not significantly different with heat-inactivated PHF. During the course of these studies, it was found that the inhibitory effect of PHF + FBS was not obtained with every PHF preparation. This is demonstrated in Table III, which shows that in the heat-inactivation experiment PHF + FBS was not inhibitory to outgrowth.

B. In Vivo Studies

Topical Application of Platelet Homogenate. Four pigs were used in this experiment. Treatments (platelet homogenate or vehicle) were applied immediately after wounding and each subsequent day. Approximately 50 ul/wound was applied with a plastic pipette to cover the wound bed and incubated in place for 5 min. Five wounds from each group were sampled by excision each day for Days 2-7 after wounding and evaluated for epidermal resurfacing and 2 wounds/day for Days 1-7 were analyzed for collagen biosynthesis. The results for epidermal resurfacing are summarized in Table IV. The results of dermal collagen

biosynthesis are shown in Table V. This experiment indicates that there is no decrease in epidermal healing time and no increase in dermal collagen biosynthesis with daily 5 min topical application of platelet homogenate. These results are discussed below. One of the possible explanations for the absence of a healing effect is that the method of applying platelet homogenate was not effective. Therefore the following experiment was designed to improve the method of exposing wounds to platelet homogenate.

Topical Application of Platelet Homogenate with Occlusion. Two pilot studied were conducted using two animals for each study. In the first pilot the wounds were treated with 50 ul platelet homogenate (10 mg protein/ml) or vehicle and immediately covered with an adhesive film dressing. The dressing was kept in place for 6 hr then removed after which time the wounds were air-exposed. Sample wounds (5/group/day) were excised and evaluated as before. Control untreated wounds, as previously described, were also evaluated. The results for this evaluation of epidermal resurfacing are shown in Table VI A. Since only two animals were used complete data analysis was not conducted. However, the trends for epidermal healing indicate that healing was accelerated in the two treated groups (Active and Vehicle) compared with control untreated wounds. However, there was no difference in healing between Platelet Homogenate- and Vehicle-treated wounds.

In a second pilot study, the protocol was repeated using 2 hr of occlusion. The results for epidermal resurfacing are shown in Table VI B. The results of dermal collagen biosynthesis are in progress. The results of epidermal resurfacing are similar to those obtained with 6 h of occlusion. Active- and Vehicle-treated wounds did not differ and both healed slightly faster than Control Untreated wounds.

DISCUSSION

A. In Vitro Studies

Viability of Explant Cultures. Since serum is derived from whole clotted blood, it contains factors released from platelets, some of which are described in the Introduction. In this study an explant was considered viable if it demonstrated any epidermal cell outgrowth by Day 3. The results demonstrate that a factor (or factors) present in platelets (PHF) supported the viability of epidermal cells from explants, since the cultures incubated with PHF alone were viable for at least a week. The % viable explants (Table I) obtained with PHF (45-69%) was similar to the % viable with 5% FBS (67%). Cultures grown with PHF + FBS showed lower overall viability (23-37%), suggesting that the combination may be less favorable for explant viability. Some outgrowth was observed without PHF or FBS (2% viable). The fact that any outgrowth occurred in unsupplemented medium may be attributed to residual nutrients within the explant tissue. However, this growth occurred in only 2 of 120 specimens, was not as vigorous as with supplemented media, and did not continue beyond Day 5. The factors in PHF responsibile for epidermal cell viability is probably not PDGF since PDGF alone did not produce viable explant outgrowth (Table II).

Initiation of Epidermal Cell Outgrowth. These experiments show that epidermal cell migration was initiated in serum-free medium containing PHF. Since the initiation of explant outgrowth requires attachment and spreading, PHF contains factors that allowed or facilitated these events. However, PHF did not initiate outgrowth as effectively as 5% FBS, based on the outgrowth measurements of Day 3 (Table I). The radius of outgrowth was greater in the 5% FBS control group than in any PHF group, and the radius of outgrowth on Day 3 was inversely proportional to the PHF concentration (See Figure 1). This finding may indicate that the outgrowth initiation factor in PHF was present in such high concentrations that it inhibited initiation. This observation is discussed below with respect to inhibition of outgrowth. Alternatively the observed delay in the onset of outgrowth may have been due to the absence in PHF of a factor for outgrowth initiation that was present in FBS; therefore, explants cultured in PHF were slower to begin growing.

Stimulation of Epidermal Cell Outgrowth. PHF contains a factor that stimulated the rate of outgrowth in a concentration-dependent manner (Figure 2). This stimulatory factor is probably different from PDGF since the results in Table II show that explants did not grow in PDGF-supplemented medium without FBS. This conclusion is also supported by the work of Heldin et al. (5) who demonstrated that an epithelial cell line lacks cell surface receptors for PDGF. Aso et al. (6) reported that culture medium containing platelet "factor" plus plasma increases the size and number of colonies in keratinocyte cultures compared with platelet-poor plasma alone; but the "factor" prepared by Aso et al. was obtained from thrombin-treated platelets and presumably contained PDGF and all other products released from the platelet following thrombin activation. It is possible that PDGF may act synergistically with another factor present in PHF (and serum) to stimulate epidermal cell outgrowth. This idea is supported by the results in Table II which show that PDGF + FBS was stimulatory for epidermal outgrowth.

In addition to PDGF, other blood-derived factors have been identified (See Introduction) and it is possible that they are responsible for some of the effects on epidermal cell outgrowth. The most likely candidates for involvement are Epidermal Growth Factor (27), Fibronectin (59), Epibolin (28) and Serum Spreading Factor (29). Since the PHF stimulatory effect is stable to heat it is probably not Fibronectin, Epibolin or Serum Spreading Factor which are heat labile. It is possible that at least part of the stimulatory effect of PHF on explant outgrowth is due to Epidermal Growth Factor which is heat-stable.

Inhibition of Epidermal Cell Outgrowth. PHF and FBS together resulted in inhibition of viability, total outgrowth and rate of outgrowth (Table I, Figures 1,2). The combination of FBS (which contains platelet factors) with PHF may have resulted in inhibition by raising the concentration of a platelet component that is involved in epidermal cell attachment and spreading. The observed inhibition may have resulted from 1) excess amount of a stimulatory factor that actually retarded outgrowth at higher concentrations, 2) increased

amount of an inhibitor to an effective concentration for blocking or decreasing outgrowth, 3) the generation of inhibitory products by combining PHF and FBS, or 4) increased amount of a cytotoxin that killed a portion of the epidermal cell population. The fourth possibility is eliminated by microscopic evidence which showed no significant cell death but rather a piling up of cells in the epidermal sheet that did not exhibit migratory morphology but were rounded and poorly spread (Figure 3); the epidermal sheet was multilayered and it appeared that migration was inhibited. The first possibility listed above is a plausible one since such an effect has been observed in SV40-transformed 3T3 fibroblast cultures incubated with supra-optimal concentrations of Serum Spreading Factor (27). By combining PHF and FBS the total concentration of an attachment factor may have exceeded the range which promotes cell attachment and spreading and the cells would then appear rounded and poorly spread. This inhibition may be related to the observed decrease in outgrowth initiation obtained with higher PHF concentrations since initiation requires cell attachment and spreading. However, the observed decrease in outgrowth may in fact reflect an actual acceleration of another process, such as cell division or differentiation; which would result in vertical rather than lateral growth. It is also possible that a PHF component combined with a serum component to generate an inhibitior (No. 3 above). Since this inhibitory effect is not dependent on the PHF concentration, it may be a non-competitive type of inhibition or the PHF concentrations tested may be outside the concentration-dependent range. This inhibitory effect was not observed in every preparation of PHF suggesting that it either was not universally present in platelets or was very labile and lost during processing in the blood bank or the laboratory. Another explanation is that the effect may have been an experimental artifact. Knowledge of the mechanism of PHF inhibition will require purification and examination of the individual aspects of epidermal outgrowth--attachment, spreading, migration, mitosis, maturation--and their responses to platelet subfractions.

B. In Vivo Studies

Daily topical treatment with Platelet Homogenate did not affect epidermal resurfacing (Table IV) compared to Vehicle-treated or Untreated wounds. Nor was dermal healing as measured by collagen biosynthesis stimulated by the application of Platelet Homogenate compared with Vehicle alone. Since the period of exposure (5 min) may have been too short to be effective, treatments were next applied in a single dose on Day 0 with occlusion to extend the exposure period.

With 6 hr of occlusion both the Active- and Vehicle-treated wounds showed enhanced healing compared with Untreated (air exposed) wounds. However, there was no difference in healing between the two treatments. Apparently, occlusion alone was responsible for accelerated epidermal healing. Even with 2 hr of occlusion there was accelerated epidermal healing in both treatment groups but no additional enhancement with platelet homogenate. Therefore, in these two pilot studies an effect by platelet homogenate may have been masked by the occlusion effect. This finding indicates that brief occlusion (2-6 hrs) may be sufficient to promote healing. It has already been established that occluded wounds

heal faster than air-exposed wounds (36). These data suggest that in order to achieve this beneficial effect, occlusion immediately after wounding may be necessary and that a relatively brief period of occlusion (less than a day) may be sufficient.

In addition to the possible masking of a platelet effect, it is also possible that dermal fibroblasts in the wound bed and epidermal cells in the reservoirs and wound margins were already maximally stimulated by endogenous platelet-derived factors. One way to consider this second possibility would be to test whether "resting" fibroblasts in non-wounded tissue can be stimulated by intradermal injection of platelet homogenate. Such a study is now in progress. Different concentrations of platelet homogenate should also be tested to find the optimal range.

CONCLUSIONS

This investigation has shown that Platelet-derived components support the growth of epidermal cells *in vitro* in explant cultures, suggesting that platelet may have a function in epidermal healing. Thus far, attempts to demonstrate an effect *in vivo* have not been successful but a concerted effort is being made to solve problems in the method of evaluation.

RECOMMENDATIONS

1. Platelet Homogenate Fraction should be further fractionated and the explant culture model utilized to identify the active components.

2. These components should be compared with known growth factors, especially Epidermal Growth Factor and Serum Spreading Factor (or Epibolin).

3. A purified active fraction should then be evaluated *in vivo* using the porcine wound model. A purified fraction may be compounded with a neutral topical vehicle for more efficacious treatment of wounds.

4. A treatment which has been successful in stimulating wound healing in the animal model should be evaluated in the clinic with human volunteers.

These recommendations are addressed in our Research Proposal "The Effects of Platelet Factors on Epidermal Resurfacing during the Repair of Partial Thickness Wounds" which is a continuation of this investigation.

REFERENCES

1. Krizek, T.J., Robson, M.C. and Wray, R.C. (1968). The functional anatomy of skin. In: The Management of Trauma. (Ed. by W. Ballinger et al.) W.B. Saunders Co., Phila. Pa., p. 650-702.

2. Gould, B.S. (1968). Collagen biosynthesis. In: Treatise on Collagen. Vol. 2A Biology of Collagen. (edited by B.S. Gould) London Academic Press, London, England, p. 139-188.

3. Grant, M.E. and Prockop, D.J. (1972). The biosynthesis of collagen. N. Engl. J. Med. 286, 4:194-199.

4. Pillsbury, D.M., Shelley, W.B. and Kligman, A.M. (1965). Corium and subcutaneous tissue. In: Dermatology. W.B. Saunder Co., Phila. Pa., p. 67.

5. Pillsbury, D.M., Shelley, W.B. and Kligman, A.M. (1965a). Hair and the glands of the skin. In: Dermatology. W.B. Saunders Co., Phila. Pa., p. 40.

6. Balk, S.D. (1971). Calcium as a regulator of the proliferation of normal, but not transformed, chicken fibroblasts in a plasma-containing medium. Proc. Natl. Acad. Sci. USA, 68:271-275.

7. Ross, R., Glomset, J., Kariya, B., and Harker, L. (1974). A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. USA, 71:1207.

8. Kohler, N. and Lipton, A. (1974). Platelets as a source of fibroblast growth-promoting activity. Exp. Cell Res. 87:297-301.

9. Harker, L.A., Ross, R., Slichter, S.J. and Scott, R.S. (1976). Homocystine-induced arteriosclerosis: The role of endothelial cell injury and platelet response in its genesis. J. Clin. Invest. 58:731-771.

10. Westermark, B., and Wasteson, A. (1983). Biochemistry of platelet-derived growth factor. In: Growth and Maturation Factors (Guroff, G., ed.) John Wiley and Sons, New York. 1:73.

11. Ross, R. and Vogel, A. (1978). The platelet-derived growth factor. Cell 14:203-210.

12. Deuel, T.F., Huang, J.S., Proffitt, R.T., Baenziger, J.U., Chang, D. and Kennedy, B.B. (1981). Human platelet-derived growth factor. Purification and resolution into two growth factor. J. Biol. Chem. 256:8896-8899.

13. Antoniades, H.N. (1981). Human platelet-derived growth factor (PDGF): Purification of PDGF-I and PDGF-II and separation of their reduced subunits. Proc. Natl. Acad. Sci. USA, 78:7314-7317.

14. Grotendorst, G.R., Seppa, H.E.J., Kleinman, H.K. and Martin, G.R. (1981). Attachment of smooth muscle cells to collagen and their migration toward platelet-derived growth factor. Proc. Natl. Acad. Sci. USA 78:3669-3679.

15. Rutherford, R.B. and Ross, R. (1976). Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. J. Cell Biol. 69:196-203.

16. Eichner, J., Cardinale, G. and Fuller, G. (1976). Effects of platelets on collagen synthesis in cultured rabbit aortic smooth muscle cells. Fed. Proc. 35:679.

17. Burke, J.M. and Ross, R. (1977). Collagen synthesis by monkey arterial smooth muscle cells during proliferation and quiescence in culture. Exptl. Cell Res. 107:387-395.

18. Heldin, C.H., Westermark, B., and Wasteson, A. (1981) Specific receptors for platelet-derived growth factor on cells derived from connective tissue and glia. Proc. Natl. Acad. Sci. USA, 78:3664-3668.

19. Aso, K., Konda, S. and Amano, M. (1980). Platelet-dependent serum factor that stimulates the proliferation of epidermal cell in vitro. In: Current Prolbems in Dermatology Vol 10 biochemistry of Normal and Abnormal Epidermal Differentiation (Bernstein, I.A. and Makoto, S., eds.) S. Karger, New York pp. 83-89.

20. Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.-H., Huang, J.S. and Devel, T.F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein $p28^{sis}$ of simian sarcoma virus. Nature 304:35-39.

21. Doolittle, R.F., Hunkapil, M.W., Hood, L.G. Devare, S.G., Robbins, K.L., Aaronson, S.A. and Antoniades, H.N. (1983) Simian sarcoma virus oncogene, V-sis is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221:275-277.

22. Paul, D., Niewiarowski, S., Varma, K.G., Rucininski, B., Rucker, S., and Lange, E. (1980). Human platelet basic protein associated with antiheparin and mitogenic activities. Purification and partial characterization. Proc. Natl. Acad. Sci. USA, 77:5914-5918.

23. Castor, C.W., Ritchie, J.D., Scott, M.E., and Whitney, S.L. (1977). Connective tissue activation. XI stimulation of glycosamino glycan and DNA formation by a platelet factor. Arthrit. Rheum. 20:859-868.

24. Heldin, C.-H., Wasteson, A. and Westermark, B. (1977). Partial purification and characterization of platelet factors stimulating the multiplication of normal human glial cells. Exptl. Cell Res. 109:429-437.

25. Eastment, C.T. and Sirbasku, D.A. (1980). Human platelet lysate contains growth factor activities for established cell lines derived from various tissues of several species. In Vitro 16:694-705.

26. Kepner, N. and Lipton, A. (1981). A mitogenic factor for transformed fibroblasts from human platelets. Cancer Res. 41:430-432.

27. Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J. Biol. Chem. 237:1555-1562.

28. Stenn, K.S. (1981). Epibolin: A protein of human plasma that supports epithelial cell movement. Proc. Natl. Acad. Sci. USA, 78:6907-6911.

29. Barnes, D.W., Silnutzer, J., See, C. and Shaffer, M. (1983). Characterizatoin of human serum spreading factor with monoclonal antibody. Proc. Natl. Acad. Sci. USA 80:1362-1366.

30. Fisher, S.M., Viaje, A., Mills, G.D., and Slaga, T.J. (1980) Explant methods for epidermal cell culture. Meth. Cell. Biol. 21A:207-227.

31. Holladay, L.A., Savage, C.R., Cohen, S. and Puett, D. (1976). Conformation and unfolding thermodynamics of epidermal growth factor and derivatives. Biochemistry 15:2624-2633.

32. Savage, C.R. and Cohen, S. (1972). Epidermal growth factor and a new derivative. Rapid isolation procedures and biological and chemical characterization. J. Biol. Chem. 247:7609-7611.

33. Savage, C.R., Hash, J.H. and Cohen, S. (1973). Epidermal growth factor. Location of disulfide bonds. J. Biol. Chem. 248:7669-7672.

34. Oka, Y. and Orth, D.N. (1983). Human plasma epidermal growth factor/-urogastrone is associated with blood platelets. J. Clin. Invest. 72:249-259.

35. Hammar, H. and Halpin, K. (1981). Epidermal cell growth. In: The Epidermis in Disease, (Marks, R. and Christophers, E. eds.) London, MTP Press, pp. 243-271.

36. Eaglstein, W.H. and Mertz, P.M. (1978). New method for assessing epidermal wound healing: the effects of triamcinolone acetonide and polyethylene film occlusion. J. Invest. Dermatol. 71:382-384.

37. Winter, G.D. (1972). Epidermal regeneration of the domestic pig. In: Epidermal Wound Healing. (Ed. by H.J. Maibach and D.T. Rovee) Year Book Medical Publishers, Inc. Chicago, Ill., p. 71-112.

38. Diegelmann, R.F., Rathkopf, L.C. and Cohen, I.K. (1975). Measurement of collagen biosynthesis during wound healing. J. Surg. Res. 19:239-243.

39. Geronemus, R.G., Mertz, P.M. and Eaglstein, W.H. (1979) Wound healing: The effects of topical antimicrobial agents. Arch. Dermatol. 115:1311-1314.

40. Eaglstein, W.H. and Mertz, P.M. (1980) Petrolatum interference with the oxidation of arachidonic acid. Brit. J. Derm. 103:257.

41. Alvarez, O.M., Mertz, P.M. and Eaglstein, W.H. (1982) The effect of the proline analogue L-azetidine-2-carboxylic acid (LACA) on epidermal and dermal wound repair. J. Plast. Reconst. Surg. 69(2):284-289.

42. Goldblum, O.M., Alvarez, O.M., Mertz, P.M. and Eaglstein, W.H. (1983) Dimethyl sulfoxide (DMSO) does not affect wound healing. Proc. Soc. Exper. Bio. Med. 172:301-307.

43. Alvarez, O.M., Mertz, P.M. and Eaglstein, W.H. (1983) Benzoyl peroxide and epidermal wound healing. Arch. Derm. 119:222-225.

45. Alvarez, O.M., Mertz, P.M., Smerbeck, R.V. and Eaglstein, W.H. (1983) The healing of superficial skin wounds is stimulated by external electrical current. J. Invest. Dermatol. 81:144-148.

46. Rovee, D.T., Lowell, J. and Miller, C.A. (1967). Tensiometric analysis of epidermal healing in the plantar skin of guinea pigs. J. Invest. Dermatol. 48:266-267.

47. Leitzel, K., Cano, C. Marks, J. and Lipton, A. (1982). Failure of nerve growth factor to enhance wound healing in the hamster. J. Neurosci. Res. 8:413-417.

48. Cohen, I.K., Clark, D.M. and Diegelmann, R.F. (1979). Onset and localization of collagen synthesis during wound healing in open rat skin wounds. Proc. Soc. Exp. Bio. Med. 160:458-462.

49. Viljanto, J. (1964). Biochemical basis of tensile strength in wound healing: An experimental study with viscose cellulose sponges in rats. Acta Chir. Scand. (Suppl.) 333:1-101.

50. Franklin, J.D. and Lynch, J.B. (1979). Effects of topical applications of epidermal growth factor on wound healing. Plast. Recon. Surg. 64:766-770.

51. Rovee, D.T., Kurowsky, C.A. and Labun, J. (1972) Local wound environment and epidermal healing. Mitotic response. Arch. Dermatol. 106:330-334.

52. Fogdestam, I. and Gottrup, F. (1980) Biomechanical methods in wound healing research with special reference to skin and gastro-intestinal tract. In: Biology of Collagen (Viidik, A. and Vuust, J., eds.) Academic Press, New York pp. 363-371.

53. Knighton, D.R., Hunt, T.K., Thakral, K.K. and Goodson, W.H. (1982). Role of platelets and fibrin in the healing sequence. Ann. Surg. 196:379-388.

54. Salganicoff, L., Hebda, P.A., Yandrasitz, J. and Fukami, M.H. (1975). Subcellular fractionation of pig platelets. Biochim. Biophys. Acta 385:394-411.

55. Bradford, M.D. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

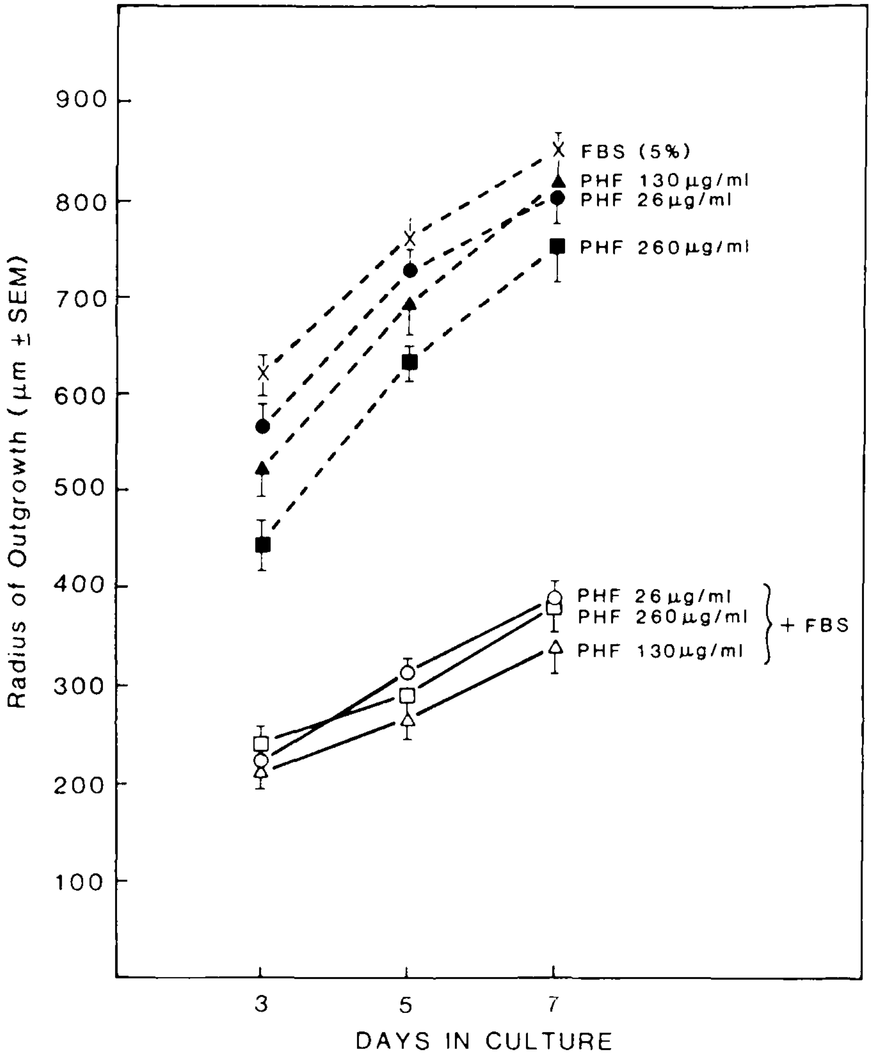
56. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

57. Halprin, K.M., Lueder, M. and Fusenig, M.E. (1979) Growth and differentiation of postembryonic mouse epidermal cells in explant cultures. J. Invest. Dermatol. 72:88-98.

58. Sokal, R.R. and Rohlf, F.J. (1981) Biometry. The principles and practice of statistics in biological research (2nd ed.). W.H. Freeman and Co., San Francisco.

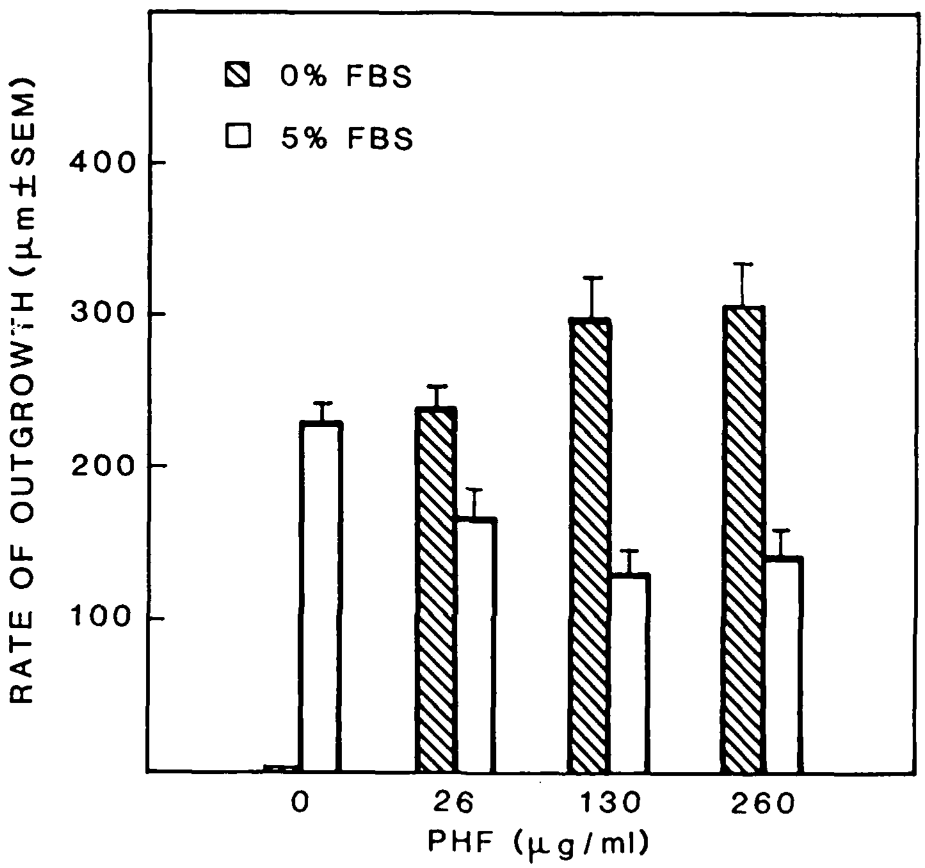
59. Yamada, K.M. and Olden, K. (1978) Fibronectins - adhesive glycoproteins of cell surface and blood. Nature (London) 274:179-184.

FIGURE 1: EPIDERMAL CELL OUTGROWTH FROM EXPLANTS



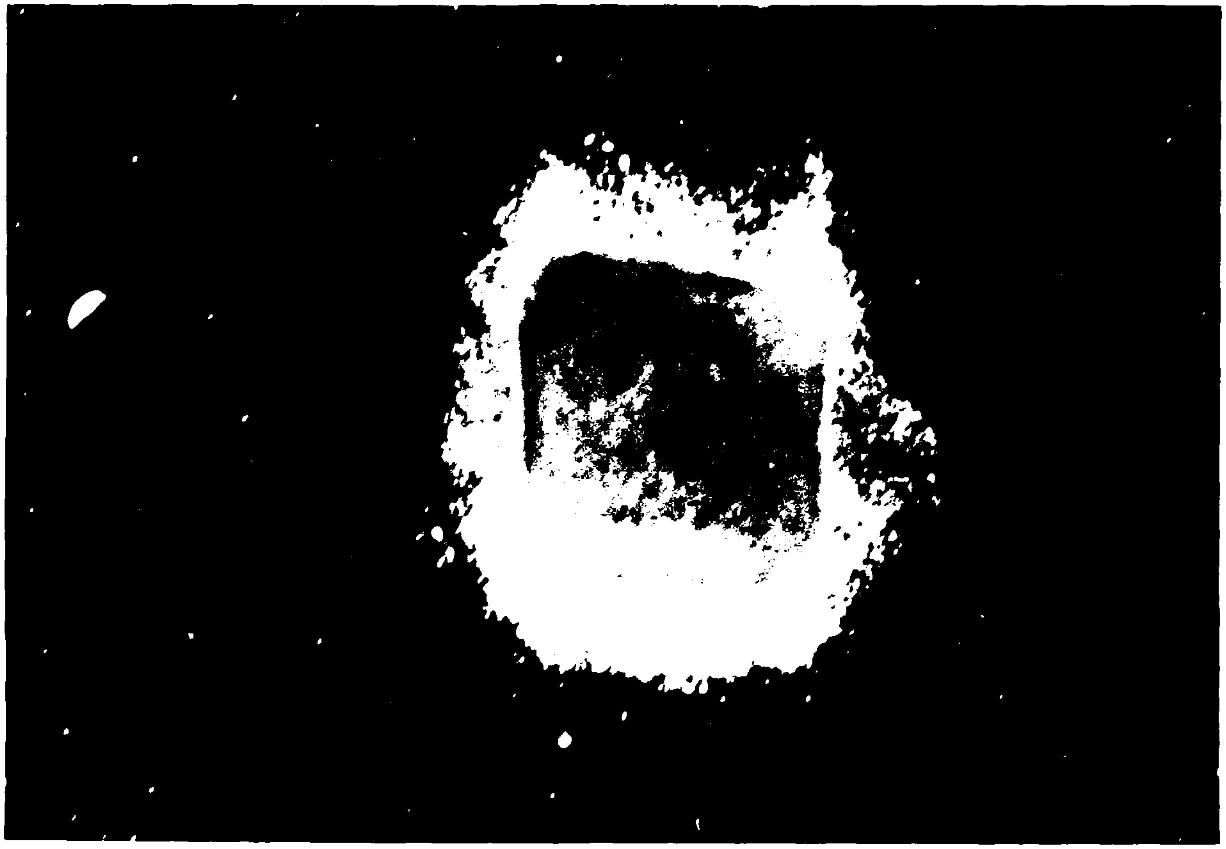
Outgrowth measurements on Day 3 were tested for significant differences using Student's t-test, with the following results: FBS vs. PHF (all groups) $p < 0.05$, PHF (26 ug/ml) vs. PHF (130 ug/ml) not significant, $p > 0.05$, PHF, (130 ug/ml) vs. PHF (260 ug/ml) $p = 0.05$, PHF + FBS (all groups) vs. PHG or FBS alone $p = 0.001$.

FIGURE 2: DIFFERENCES IN OUTGROWTH RATES BETWEEN DAYS 3 AND 7



Rates of outgrowth were tested for significant differences using Student's t-test, with the following results: FBS vs. PHF (26 ug/ml) not sig icant, p > 0.05, PHF (26 ug/ml) vs. PHF (130 or 260 ug/ml) p < 0.05, PHF (130 ug/ml) vs. PHF (260 ug/ml) not significant, p > 0.05, PHF + FBS (all groups) vs. PHF or FBS alone p = 0.001.

FIGURE 3: PHOTOMICROGRAPHS OF EXPLANTS ON DAY 7

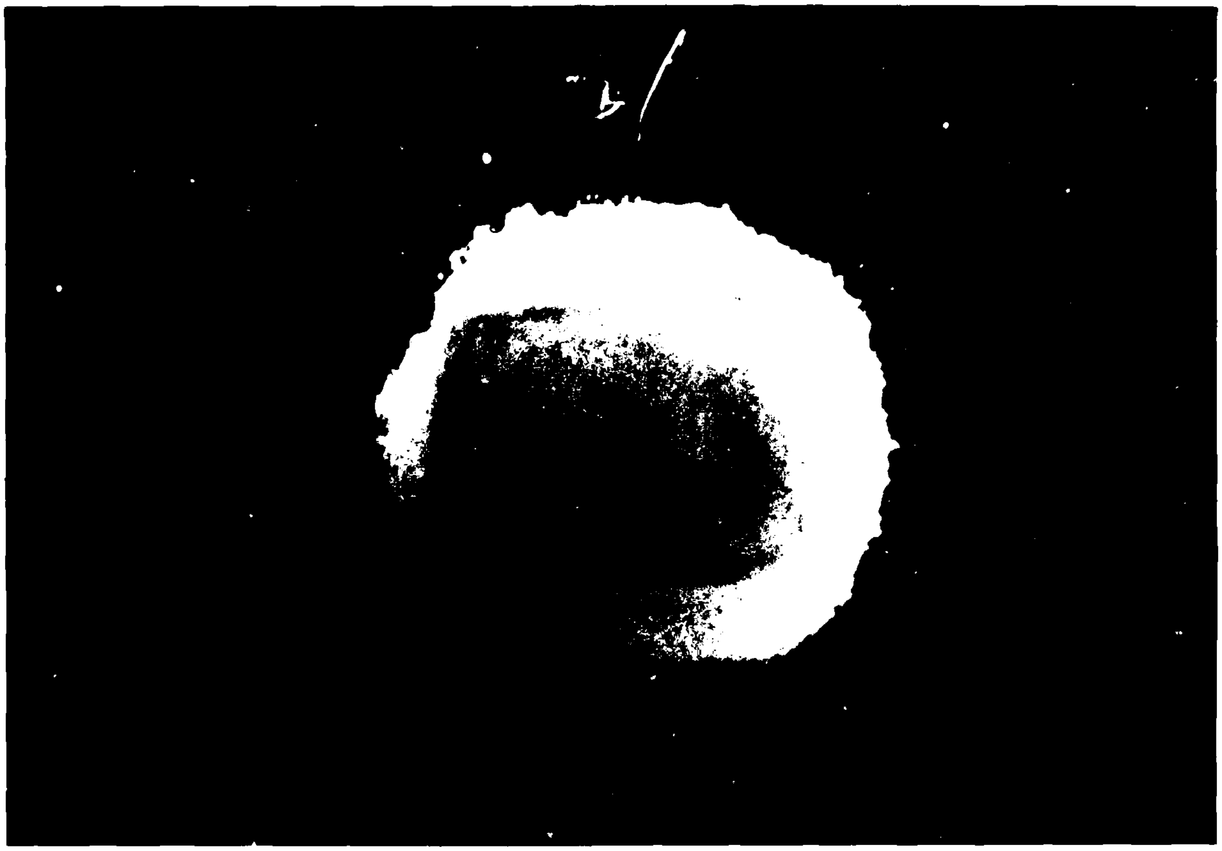


A. Explant cultured in 26 ug/ml PHF under dark field microscopy (X40). The original explant (square piece of skin) is seen surrounded by the epidermal sheet (outgrowth). Note gradation in color of epidermal sheet indicating gradual thinning to a monolayer at the growing (outer) edge.

FIGURE 3: PHOTOMICROGRAPHS OF EXPLANTS ON DAY 7

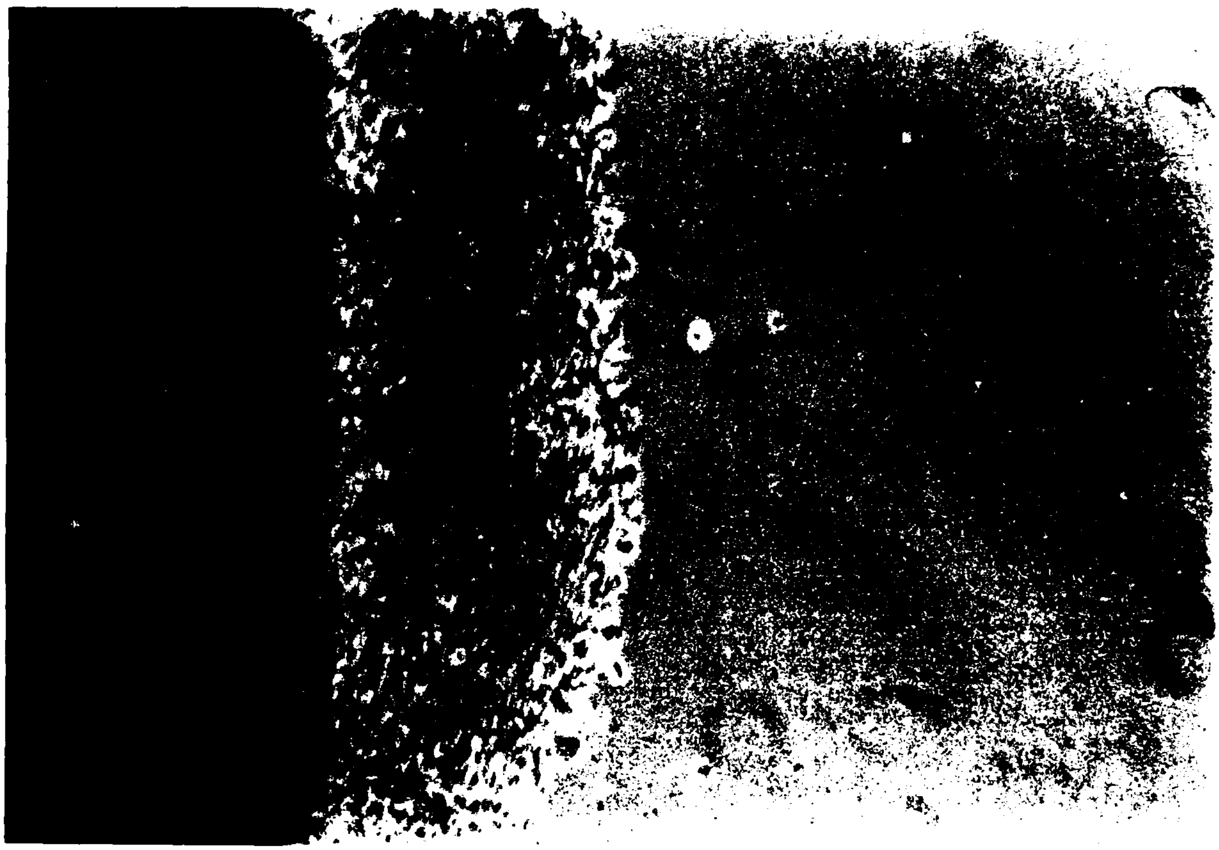
B. Same explant as in 3.A. shown in phase contrast at 100X. Note thick, multilayerd sheet near the explant (left) tapering to monolayer of "stretched" cells at the growing edge (right).

FIGURE 3: PHOTOMICROGRAPHS OF EXPLANTS ON DAY 7



C. Explant cultured in 130 ug/ml PHF + 5% PBS under dark field microscopy (X40). Note uniformly thick epidermal sheet.

FIGURE 3: PHOTOMICROGRAPHS OF EXPLANTS ON DAY 7



D. Same explant as in 3.C. shown in phase contrast at 100X. Note absence of flattened and "stretched" cells at growing edge (right). All the cells are rounded and piled up.

TABLE I: THE EFFECTS OF PLATELET HOMOGENATE FRACTION (PHF) AND FETAL BOVINE SERUM (FBS) ON EXPLANT OUTGROWTH.

0% FBS

Group No.	PHF (ug/ml)	% Viable[1]	Outgrowth (um ± SEM) Day 3	Day 5	Day 7
1	0	2%	366 ± 79	516 ± 17	522 ± 11
2	26	69%	566 ± 24	728 ± 24	804 ± 25
3	130	45%	522 ± 26	692 ± 29	818 ± 32
4	260	55%	445 ± 26	633 ± 18	751 ± 33

5% FBS

Group No.	PHF (ug/ml)	% Viable[1]	Outgrowth (um ± SEM) Day 3	Day 5	Day 7
5	0	67%	620 ± 21	761 ± 23	851 ± 20
6	26	30%	224 ± 13	314 ± 17	390 ± 20
7	130	26%	210 ± 14	265 ± 18	340 ± 24
8	260	37%	242 ± 17	290 ± 15	383 ± 24

[1] % Viable = n/T x 100% (n = number of explants growing on Day 3 and T = total number of explants prepared, 120)

TABLE II: THE EFFECT OF PLATELET-DERIVED GROWTH FACTOR (PDGF) ON EXPLANT OUTGROWTH

PDGF[2] (units/ml)	Relative Epidermal Cell Outgrowth[1] 0% FBS	5% FBS
0	0	100 (±10)
0.2	0	115 (± 7)[3]
1.0	0	120 (± 4)[4]
5.0	0	124 (± 2)[4]

[1] Results are expressed relative to 5% FBS, % outgrowth (± SEM).

[2] 1 unit/ml PDGF = the amount which evokes a response in fibroblasts equal to that of 5% FBS.

[3] Not significantly different from FBS control ($p > 0.05$).

[4] Significantly different from FBS control ($p < 0.05$).

TABLE III: THE EFFECT OF HEAT INACTIVATION ON PHF-SUPPORTED EXPLANT OUTGROWTH

Supplement	Relative Epidermal Cell Outgrowth[1] before heat	after heat
FBS (5%)	100 (± 9)	--
PHF (26 ug/ml)	88 (± 6)[2]	81 (±7)[2]
PHF + FBS	114 (±10)[2]	122 (±7)[2]

[1] See Table II.

[2] Not significantly different from FBS control ($p > 0.05$).

TABLE IV: THE EFFECT ON EPIDERMAL RESURFACING OF TOPICALLY APPLIED PLATELET HOMOGENATE

Group	Treatment	HT_{50} [1]
Active	3×10^{10} platelets/ml in PIM[2]	5.4 days
Vehicle	PIM	5.4 days
Untreated	---	5.4 days

[1] HT_{50} = Healing Time 50 - time required for 50% of the wounds to heal.

[2] PIM - Platelet Incubation Medium - 100 mM KCl, 50 mM Tris-HCl, 5 mM $MgCl_2$ 1 mM EDTA, pH 7.4.

TABLE V. THE EFFECT ON DERMAL COLLAGEN BIOSYNTHESIS OF TOPICALLY APPLIED PLATELET HOMOGENATE

Group	Treatment	Relative Collagen Biosynthesis[1] Relative Units (SEM)
Active	3×10^{10} platelets/ml in PIM[2]	1.68 (0.37)[3]
Vehicle	PIM[2]	1.96 (0.52)[3]
Untreated	---	1.00

[1] The mean values from four (4) animals of the % RCB on post-wounding Day 2 (the time during which collagen biosynthesis is stimulated) have been normalized to Untreated wounds to correct for animal to animal variation.

[2] PIM - Platelet Incubation Medium - 100 mM KCl, 50 mM Tris-HCl, 5 mM $MgCl_2$ 1 mM EDTA, pH 7.4

[3] Not significantly different ($p > 0.05$)

TABLE VI: EFFECTS ON EPIDERMAL RESURFACING OF TOPICAL PLATELET HOMOGENATE AND OCCLUSION

A. Pilot Study #1 - Six Hours of Occlusion (Two Animals)

No. of Wound Healed/No. of Wounds Evaluated

Treatment	Day 3	Day 4	Day 5	Day 6	Day 7
Untreated	0/10	1/10	2/7	6/10	10/11
	(0%)	(10%)	(29%)	(60%)	(91%)
Vehicle	0/9	4/10	6/9	9/9	8/8
	(0%)	(40%)	(67%)	(100%)	(100%)
PHF	0/10	0/10	5/7	9/9	9/9
	(0%)	(0%)	(72%)	(100%)	(100%)

B. Pilot Study #2 - Two Hours of Occlusion (Two Animals)

No. of Wounds Healed/No. of Wounds Evaluated

Treatment	Day 3	Day 4	Day 5	Day 6	Day 7
Untreated	0/10	0/11	8/9	10/10	7/7
	(0%)	(0%)	(89%)	(100%)	(100%)
Vehicle	0/8	9/10	9/9	8/9	10/10
	(0%)	(90%)	(100%)	(89%)	(100%)
PHF	0/9	6/8	10/10	10/10	7/7
	(0%)	(75%)	(100%)	(100%)	(100%)

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Since keratinocyte growth may be enhanced by hormones or matrix proteins, we extracted placenta and tested the extracts for growth-promoting activity. Human placenta was extracted with neutral saline, 2 M NaCl, 4 M urea, or 2 M KSCN and the extract dialyzed against water, centrifuged to remove precipitates, and lyophilized. Cells from sub-confluent human keratinocyte cultures were plated in MCDB 153 medium supplemented with insulin (5 μg/ml), hydrocortisone (0.4 μg/ml), transferrin (5 μg/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), and 0.5 mg per ml or less of the extracts. The most potent growth-promoting activity, detectable at 5 μg per ml, was present in a neutral saline Ca^{++}-Mg^{++}-free extract containing 1 mM EDTA and 1 mM PMSF. Cell counts of trypsinized cells showed greater than 100-fold enhanced cell number after 9 days at inoculation densities of 10^4 cells per 35 mm dish and markedly enhanced plating efficiency at lower inoculation densities. At higher inoculation densities, keratinocytes became confluent earlier in the presence of the extract. Morphology of colonies differed from that of cells grown in dishes treated with purified agents with cell spreading activity such as collagen type I or IV or fibronectin, and cell number at confluence was higher in the presence of the extract than after plating on dishes with spreading agents. Addition of epidermal growth factor altered morphology of colonies and further enhanced growth. Human placenta contains a soluble keratinocyte growth promoting activity which is probably not a matrix molecule and which is distinct from epidermal growth factor.

4. Role of Fibronectin in Keratinocyte Spreading, Attachment and Movement. E. J. O'Keefe, D. T. Woodley, R. E. Payne, and N. T. Russell, Dept. of Dermatology, University of North Carolina, Chapel Hill, NC.

We have previously found that cultured human keratinocytes synthesize and deposit fibronectin on the culture surface. In this study we examined the role of fibronectin in the spreading, attachment and movement of keratinocytes. Cells subcultured on tissue culture plastic treated with matrix proteins were fixed after 4 h and assayed for spreading under phase contrast optics. The percentage of attached cells was determined by counting cells released by trypsin 4 h after plating. Movement was assessed by observing displacement of particulate gold salts on the culture surface. Less than 10% of cells adherent to tissue culture plastic were spread, but 60–98% of cells were spread on fibronectin or collagen types I or IV in the presence or absence of cycloheximide. After 24 h in the presence of cycloheximide, spreading was similar with or without matrix proteins. Antibody to fibronectin prevented spreading by fibronectin, but not by collagen type IV. In contrast to cell spreading, attachment was increased only moderately by fibronectin (41%) or collagen type IV (10%) and was decreased by collagen type I (27%), indicating that spreading and attachment may reflect different types of cell-matrix interactions. Increased cell density or collagen type IV enhanced cell movement. Although exogenous fibronectin had little effect on cell movement, fibronectin deposited by the cells and detected by immunofluorescence correlated with displacement of gold particles, indicating that a keratinocyte deposits fibronectin along the path it traverses. Fibronectin promotes spreading and attachment of cultured human keratinocytes and is associated with their paths of movement.

5. Human Keratinocyte Adhesion and Phagocytosis Promoted by Fibronectin. A. Takashima and F. Grinnell, Dept. of Cell Biology, University of Texas Health Science Center, Dallas, TX.

Early passage keratinocytes from human foreskin or cadaver skin were tested for attachment and spreading on human plasma fibronectin (pFN)-coated culture dishes and binding and phagocytosis of pFN-coated latex beads. When cells were incubated on dishes coated with 20 μg/ml pFN, 15% of the cells attached in 1 hour, and 80% of the attached cells were spread. On serum albumin (BSA)-coated dishes, however, only 1% of the cells attached, and there was little if any spreading. In dose-response experiments, maximal spreading of keratinocytes occurred with 20 μg/ml pFN, but maximal spreading of human fibroblasts required only 2–5 μg/ml pFN. Keratinocytes incubated in suspension with 0.76 μm latex beads were found to bind pFN-coated beads but not BSA-coated beads, and much more bead binding occurred at 37° than at 4°. Based on cell morphology, basal keratinocytes appeared to bind beads better than differentiated cells. Except at early times most of the bound beads could not be removed by trypsin treatment suggesting that they were internalized. This possibility was confirmed since most of the pFN-coated beads (all following trypsinization) were detectable by indirect immunofluorescence with anti-pFN antibodies, only if the cells were permeabilized. Also, the internalized beads appeared to be in a perinuclear distribution. Finally, in both adhesion and phagocytosis assays the cells were confirmed as keratinocytes by indirect immunofluorescent staining with anti-keratin antibodies.

6. The Effect of Platelet Factors on Epidermal Cell Outgrowth in Skin Explants. P. A. Hebda and W. H. Eaglstein, Dept. of Dermatology, University of Pittsburgh School of Medicine, Pittsburgh, PA.

We previously reported that a commercial preparation of Platelet-Derived Growth Factor (PDGF) enhances epidermal cell outgrowth from skin explants in the presence of fetal bovine serum (FBS); however, in serum-free cultures commercial PDGF did not support outgrowth. Since then we have grown skin explant cultures ± FBS and a soluble platelet homogenate fraction (PHF). The data below are a summary of an evaluation of outgrowth from a total of 1200 explants, 120 specimens in each of 10 groups. Outgrowth was measured microscopically every other day for seven days. The values below are the differences in the mean outgrowth (μm ± SE) between days 3 and 7.

FBS (%)	PHF (μg/ml) 0	26	131	262
0	0	238 ± 47	297 ± 58	358 ± 59
5	231 ± 29	166 ± 33	130 ± 36	141 ± 41

Our results show a concentration-dependent stimulation of cell outgrowth with PHF and no FBS. This stimulatory effect is heat-stable (60°C for 2 min). When explants were cultured with various concentrations of PHF and 5% FBS, slower outgrowth was observed suggesting the presence of an inhibitor. The inhibitory effect is heat-labile. We conclude that platelets contain a factor (or factors) which stimulates epidermal cells in explant cultures. Since the stimulatory factor supports growth in the absence of FBS, we believe it is not the classical PDGF. There is evidence suggesting an inhibitory factor in PHF that is active with 5% FBS. These factors, which affect epidermal cells and are probably present in wounds due to platelet aggregation, may be important in regulating epidermal cell response to wounding.

Intermission

7. The Effect of Gamma Interferon on Cultured Human Keratinocytes. Vera B. Morhenn, Brian J. Nickoloff, Thomas C. Merigan, and Teresa Y. Basham, Div. of Infectious Diseases, Depts. of Medicine and Dermatology, Stanford University School of Medicine, Stanford, CA.

Gamma interferon induces expression of HLA-DR antigen in a number of cells and cell lines: monocytes, vascular endothelium and melanoma cells. Recently, we have demonstrated that recombinant interferon-γ (r-IFN-γ) induces the expression of HLA-DR antigen on cultured human epidermal cells. To determine whether Langerhans cells (LC) mediate this expression, we depleted epidermal cell suspensions of LC and incubated these cultures with r-IFN-γ. Four days after addition of r-IFN-γ, the cultures were trypsinized and the number of viable cells/plate counted using trypan blue. Using monoclonal antibody and fluorescence activated cell sorter analysis, the cells were stained and the % fluorescent cells determined.

	cell #/plate	cells expressing HLA-DR (% above control)
Control	5.5×10^5	—
r-IFN-γ (1,000 U/ml)	2.0×10^5	65

To determine whether r-IFN-γ had these same effects on malignant keratinocytes, a human squamous cell carcinoma cell line (SCL-1) was used. The cells were incubated with r-IFN-γ for 6 days and the number of viable cells/plate and the % of cells expressing HLA-DR determined as above.

	cell #/plate	% cells expressing HLA-DR
Control	2.8×10^6	1.0
r-IFN-γ (1,000 U/ml)	0.3×10^6	38.0

The r-IFN-γ causes expression of HLA-DR antigen by normal and transformed human keratinocytes and this expression is independent of LC. Also r-IFN-γ reduces the number of keratinocytes and SCL-1 cells/plate.

8. Differentiation and Cell Cycle Specific Growth Arrest States of Normal Human Keratinocyte Stem Cells. M. R. Pittelkow, J. J. Wille, Jr., and R. E. Scott, Depts. of Dermatology and Cell Biology, Mayo Clinic and Mayo Foundation, Rochester, MN.

The relationship between proliferation and differentiation and the

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